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The following abbreviations have been used: UDPG, uridine diphosphate glucose; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; CoA, coenzyme A; EDTA, ethylene diamine tetraacetic acid; UTP, uridine triphosphate; creatine P, creatine phosphate; PEP, phosphoenol pyruvate.

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*DISSOCIATION OF HUMAN CARBONMONOXYHEMOGLOBIN AT HIGH pH**

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This communication presents a study of some of the hydrodynamic properties of the half-molecules of human carbonmonoxyhemoglobin at pH 11.0 and ionic strength 0.25, together with a demonstration that, at this pH, human carbonmonoxyhemoglobin is completely dissociated and the molecular weight of the product is one-half that of the whole molecule. The half-molecules appear to be stable when stored at pH 11 and 3° C., in contrast to their behavior in an acid environment, pH 4.5–6, in which denaturation occurs. The acid denaturation is so rapid at pH values low enough to induce complete dissociation that it has not been possible until now to study the properties of the half-molecules uncomplicated by the presence of the whole molecules. Results of a comparison of some properties of untreated hemoglobin and hemoglobin reconstituted from half-molecules by successive dialyses at pH 11 and pH 7 are also presented.

Experimental.—Crystallized carbonmonoxyhemoglobin was prepared according to the method of Drabkin.[1] This material was used in most of the sedimentation

studies. Hemoglobin obtained prior to crystallization was also employed, and no significant difference was found in the sedimentation behavior at either pH 7.0 or pH 11.0.

The following buffer solutions were used: Tris-HCl-NaCl, pH 8–9; glycine NaOH-NaCl, pH 9.6–11.6; and phosphate-NaCl, pH 11.0–11.6. The ionic strength was 0.25 in all cases. All hemoglobin solutions were dialyzed at 3° C. for 12–15 hours before the sedimentation runs.

The sedimentation and diffusion measurements were carried out in the Model E Spinco analytical ultracentrifuge, using a phase-plate schlieren diaphragm. A plastic commercial interference synthetic boundary cell[2] was used for the diffusion measurements, which were performed with an essentially static boundary at 8,000 rpm. Sedimentation velocity measurements in the pH range 10.0–11.6 were carried out in a double-cell rotor, using a 1° positive wedge. The second cell contained

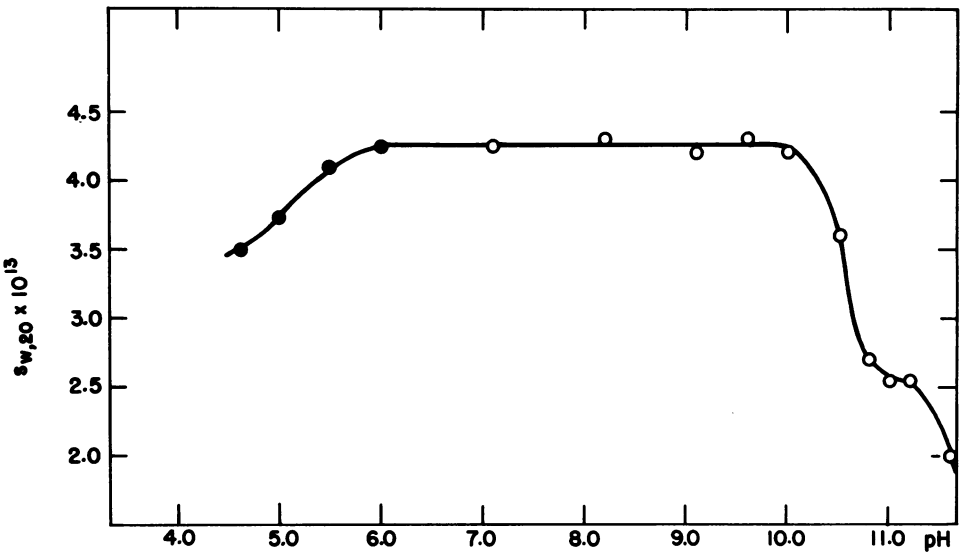

FIG. 1.—Sedimentation coefficients of human carbonmonoxyhemoglobin in the pH range 4.5–11.6. ● from Field and O'Brien (*Biochem. J.*, **60**, 656, 1955): Hb concentration: 0.71 per cent. Ionic strength of the solution: 0.1. O this study.

carbonmonoxyhemoglobin at pH 11.0 as a reference. Thus differences of 0.1 sedimentation units could be detected with considerable certainty.

The sedimentation and diffusion coefficients are expressed in the text in c.g.s. units $\times 10^{13}$ and 10^{7}, respectively.

Results and Discussion.—Table 1 shows the average sedimentation coefficients obtained in basic solutions. The trend of the values indicates that the dissociation of the hemoglobin molecule in a 1 per cent solution begins just beyond pH 10. At pH 11.0 the value of $s_{20,w}$ is 2.55. This is the value reported for horse globin[3] and also for human globin[4] at pH 7.0 and at concentrations of 1 per cent. Both these materials have half the molecular weight of the corresponding hemoglobins.[3,4] Figure 1 shows a small plateau in the s versus pH curve around pH 11.0, indicating that in this region hemoglobin is completely dissociated into two subunits. At this pH the dependence on concentration is normal, as would be expected for a fully

dissociated hemoglobin, while at pH 10.5 the results suggest the presence of incomplete dissociation (Table 1).

Molecular weight determinations at pH 11.0, using the Archibald method of approached sedimentation equilibrium,[5] gave an average value of 36,000 ± 2,000.[6] Involved in this result is the assumption that the partial specific volume does not change significantly from 0.749 ml. $g.^{-1}$.

The spreading of the boundaries at pH 10.5 and 11.0 is in accord with the above conclusions. The sedimenting boundaries after 1 hour or longer at 56,100 rpm were skewed at pH 10.5, and the spreading was greater than could be accounted for by diffusion alone. At pH 11.0 the boundaries had an approximately Gaussian shape, with less spreading than at pH 10.5 or 11.6. Thus, in plots of the standard deviation of the schlieren patterns σ versus $\sqrt{t}$, curves with an upward trend were obtained for Hb at pH 10.5 and 11.6, and a straight line at pH 11.0.

A more extended study at pH 11.0 gave the following results: the average diffusion coefficient (static) in phosphate as well as glycine-NaOH buffers is 7.6 ± 0.4; the concentration dependence lies within the limits of error; $s^\circ_{20,w}$ has the value 2.7, and $D^\circ_{20,w}$, calculated from s and M, has the value 7.4.

Moving-boundary electrophoresis at pH 11.0, $\mu = 0.1$, phosphate buffer, showed a single symmetrical boundary in the ascending and descending limb. A solution first dialyzed to pH 11 and then to pH 6.75 gave electrophoretic patterns indistinguishable from an equivalent sample dialyzed to pH 6.75 directly. A mixture of equal quantities of the foregoing materials again gave the same pattern.

The frictional ratio calculated for the subunit at pH 11.0 is 1.27, whereas this value for hemoglobin at pH 7.1 is 1.21, if $s^\circ_{20,w}$ is taken to be 4.45 and the molecular weight 66,500.[7] This slight increase in the molecular asymmetry, which corresponds, for example, to a change in the axial ratio from 3:1 to 4:1 in an ellipsoid, assuming 20 per cent hydration,[8] suggests that cleavage is along the major axis of the molecule.

The dissociation into subunits could be reversed upon neutralization. Samples of hemoglobin which had been at pH 11.0 for 12 and 36 hours, respectively, were dialyzed to pH 8.2, and the sedimentation coefficient was measured along with reference samples at the same pH. The result was 4.25.

Neither precipitation nor change in the spectrum (350–650 mμ) was observed on raising the pH to 11.0 and maintaining this pH for 36 hours. The region of the hemoglobin spectrum most sensitive to denaturation and shifting of the hemes is to be found at the maxima at 540 and 568 mμ. The ratios of the minimum at 376 mμ to the two maxima were 1.26 and 1.29 at pH 7.1 and 1.25 and 1.28 for Hb which had been at pH 11.0 for 36 hours and was redialyzed to pH 7.1.

On the other hand, the changes in the hemoglobin molecule at pH 11.6 are not reversible. The color of the solution changes, and the maxima at 540 and 568 mμ disappear slowly. Precipitation occurs on redialysis to pH 7.1, and the amount of this precipitate increases with the time of exposure to the alkali.

From the data in Table 1 and the results of Field and O'Brien[9] a pH stability diagram may be formulated (Fig. 1). This curve is not symmetrical about the isoelectric point, indicating that dissociation is not entirely the result of repulsions generated by the net negative or positive charge on the protein. Neglecting ion binding, it may be estimated[10] that at pH 6, where acid dissociation begins, the

TABLE 1

SEDIMENTATION AND DIFFUSION COEFFICIENTS OF HUMAN CARBONMONOXYHEMOGLOBIN*

pH	1 Per Cent $s_{20,w}$	0.3 Per Cent $s_{20,w}$	$D_{20,w}$
8.2	4.3	...	...
9.1	4.2	...	...
9.6	4.3		
10.0	4.2†	4.35	...
10.5	3.6†	3.4	...
10.8	2.70†	...	...
11.0	2.55	2.65	7.6 ± 0.4
11.2	2.55†	...	...
11.6	2.0†	...	...

* All measurements at $\mu = 0.25$ in buffered solutions described in text.
† Runs in double-cell rotor with a solution at pH 11 present as a reference.

molecule has a net charge of +5, whereas at pH 10 the charge is about −30. These considerations neglect binding of negative ions, which may be expected to make the situation even more asymmetric. Thus it is not likely that general net charge repulsions are solely responsible for the dissociation reaction at low pH.

An examination of the stability curve suggests that the amino acid residues critically involved in stabilizing the whole molecules are histidines, which are protonated during acid dissociation, and lysines and tyrosines, which are neutralized or ionized upon alkaline dissociation.

The temperature coefficients of the dissociation into subunits have been estimated from the temperature coefficients of the observed sedimentation coefficients at pH 5 and 10.5. The results corresponded to 0.0 ± 0.5 kcal./mole at pH 5 and −4.0 ± 0.5 kcal./mole at 10.5. These enthalpy changes are interpreted as the sums of the enthalpies of splitting the molecules plus the enthalpy changes involved in charging or discharging the critical groups. Since the number of the groups is not known, it is not possible at the present time to apportion these enthalpy changes.

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